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PRINCIPAL INVESTIGATOR: Claire I Vanpouille-Box

CONTRACTING ORGANIZATION: Weill Cornell Medical College
New York, NY 10065

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| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT In recent years, progress has been made in the development of immune-based therapy for cancer. Conceptually, these treatment strategies have the potential of harnessing the immune system to combat and eliminate cancer cells. One major obstacle to the success of immunotherapy in both human and animal studies is the development of immunologic tolerance in tumor-bearing hosts. Therefore, the immune system fails to recognize cancer cells as dangerous and actively suppresses antitumor immune responses. Identification of the underlying mechanisms and the critical players that drive tolerance to the tumor is critical to improve the therapeutic efficacy of immunotherapy. Recent data indicate that activin A, a small protein secreted by some immune cells and by breast cancer cells has immune regulatory functions that may play a key role in promoting escape of tumors from immune control. The proposed studies will test the hypothesis that activin A secreted by breast cancer cells plays a key role in suppressing antitumor immunity. The goals are to demonstrate the role of activin A produced by breast cancer cells in tumor growth and metastasis, and the potential therapeutic benefit of blocking activin A to increase the response to radiotherapy. | | | | | |
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1 INTRODUCTION

Owing to its ability to spread systematically, breast cancer remains a life-threatening tumor. Therefore, efforts in developing new treatment strategies are needed in order to eradicate metastatic breast cancer. In this respect, the activation of the immune system to elicit anti-tumor immune responses represents one of the most promising approaches that have recently demonstrated some success in other diseases. However, clinically apparent tumors have already harnessed host mechanisms to prevent immune activation and to induce an immunosuppressive microenvironment hindering immunotherapy-based treatments. As a consequence, the immune system fails to recognize cancer cells as dangerous and actively suppresses anti-tumor immune responses.

Identification of the underlying mechanisms and the critical players that drive tolerance to the tumor is critical to improve the therapeutic efficacy of immunotherapy. Recent data indicate that activin-A, a small protein secreted by some immune cells [1-3] and by breast cancer cells [4], has immune regulatory functions [5-10] that may play a key role in promoting escape of tumors from immune control. The specific hypothesis of this project is that activin-A secreted by breast cancer cells plays a key role in suppressing antitumor immunity. The goals are to demonstrate the role of activin-A produced by breast cancer cells in tumor growth and metastasis, and the potential therapeutic benefit of blocking activin-A to increase the response to radiotherapy (RT).

2 KEYWORDS

Breast cancer, metastasis, transforming growth factor-beta (TGF β) superfamily, activin-A, radiotherapy (RT), immunosuppression, immunotherapy, induced regulatory T cells (Tregs), anti-tumor immunity, abscopal effect.

3 OVERALL PROJECT SUMMARY

This award was in the transfer process from NYU to Weill Cornell for all the reporting period. As such, the PI did not complete any work on the grant during the reporting period.

4 KEY RESEARCH ACCOMPLISHMENTS

Nothing to report

5 CONCLUSION

Nothing to report

6 PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. Manuscripts submitted for publication:

1. Lay Press:
Nothing to report.

2. Peer-Reviewed Scientific Journals:

Nothing to report.

3. Invited Articles:

Nothing to report.

4. Abstracts:

- Vanpouille-Box C, Formenti SC and Demaria S. Regulation of radiation-induced in situ tumor vaccination by *TGF β superfamily*. **2016 AACR annual meeting**, USA, New Orleans, April 16-20 2016. (APPENDIX 1).
- Demaria S, Vanpouille-Box C, Hawtin RE, Fanton C. Conroy A, Evensen E, Dixit N, Barber A, Schaeue D, McBride WH, Barcellos-Hoff MH and Formenti SC. *TGF β and activin A control regulatory T cells in irradiated tumors*. **2016 AACR annual meeting**, USA, New Orleans, April 16-20 2016. (APPENDIX 2).
- Vanpouille-Box C, Formenti SC and Demaria S. *TGF β superfamily members regulate radiation-induced in situ tumor vaccination*. **2016 ASTRO annual meeting**, USA, Boston, September 25-28 2016. (APPENDIX 3).
- Vanpouille-Box C, Formenti SC and Demaria S. *ATM is essential for the radiation-induced upregulation of the immunosuppressive cytokine activin-A by breast cancer cells*. **2016 SITC Annual Meeting**, USA, National Harbor, November 9-13 2016. *Poster Presentation*. (APPENDIX 4).

b. National Meeting and Presentations:

- 2016 American Association of Cancer Research (AACR) Annual Meeting, New Orleans 16-20 April. *Poster Presentation*.
- 2016 American Society for Radiation Oncology (ASTRO) 58th annual meeting, USA, Boston, September 25-28 2016. *Oral Presentation*.
- 2016 SITC 31st Annual Meeting, USA, National Harbor, November 9-13 2016. *Poster Presentation*.

7 INVENTIONS, PATENTS AND LICENSES

Nothing to report.

8 REPORTABLE OUTCOMES

Nothing to report.

9 OTHER ACHIEVEMENTS

Awards:

- 2016 Scholar-in-Training Travel Award from the Society for Immunotherapy of Cancer.

10 REFERENCES

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10. Segerer, S.E., et al., *The glycoprotein-hormones activin A and inhibin A interfere with dendritic cell maturation*. *Reprod Biol Endocrinol*, 2008. **6**: p. 17.

11 APPENDICES

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Clinical Research (Excluding Clinical Trials)

Abstract 4986: Regulation of radiation-induced in situ tumor vaccination by TGF β superfamily members

Claire I. Vanpouille-Box, Silvia C. Formenti, and Sandra Demaria

DOI: 10.1158/1538-7445.AM2016-4986 Published 15 July 2016

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Proceedings: AACR 107th Annual Meeting 2016; April 16-20, 2016; New Orleans, LA

Abstract

Transforming Growth Factor-beta (TGF β) and activin A (actA) are members of the TGF β superfamily with overlapping as well as distinct functions in many processes including regulation of inflammation and immunity. Similar to TGF β , actA has been shown to promote the conversion of CD4+CD25- T cells into induced regulatory T cells (iTregs), and to potentiate iTregs generation by TGF β .

Many cancer cells produce actA, and we have recently found that levels of actA released in vitro by breast cancer cells are enhanced by radiotherapy (RT). Interestingly, prolonged exposure to TGF β inhibitors also resulted in enhanced actA release, consistent with a compensatory mechanism described in development (Carvalho et al., J Cell Sci, 2007). Here we tested the hypothesis that actA plays a role in regulating anti-tumor immunity induced by local tumor RT and TGF β blockade in vivo (Vanpouille-Box et al., Cancer Res 2015).

4T1 derivatives with conditional actA knockdown (4T1shActA) or non-silencing control (4T1shNS) were engineered using inducible doxycycline (DOX) plasmids and injected s.c. in BALB/c mice (day 0). ActA gene knockdown (KD) was induced by DOX at day 8. TGF β neutralizing 1D11 or isotype control antibodies were given i.p. every other day starting on day 12. RT was delivered to the primary tumor in 6Gy fractions on five consecutive days starting at day 13. Mice were followed for tumor growth and survival or euthanized at day 22 or 28 for analysis.

Consistent with in vitro data, ActA gene expression was upregulated at day 28 in 4T1 tumors of mice after prolonged TGF β blockade by 1D11. Neither 1D11 nor ActA gene KD by themselves inhibited tumor growth. However, each intervention significantly improved tumor control achieved by RT. ActA KD in mice treated with RT+1D11 prevented tumor recurrence and improved survival (RT+1D11 vs RT+1D11+shActA p = 0.026; RT+shActA vs RT+1D11+shActA p = 0.0008). Interestingly, blockade of TGF β or actA resulted in increased intratumoral Tregs (Control: 11.6%; 1D11: 26.2%, shActA: 21%) and markedly enhanced the increase in Tregs seen with RT alone (RT: 15.7%; RT+1D11: 27.5%; RT+shActA: 30.3%). In marked contrast, when both TGF β and actA were



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inhibited Tregs significantly decreased in both non-irradiated (1D11+shActA: 13.6%) and irradiated tumors (RT+1D11+shActA: 7.9% of Tregs). IFNγ production by CD8+ T cells in response to the tumor-specific AH1 peptide was significantly higher in RT+1D11+shActA treated mice compared to RT+1D11 (p = 0.045) and RT+shActA (p = 0.0147). Additionally, in RT+1D11+shActA mice expression of the activation marker CD69 was markedly increased in intra-tumoral CD8+ PD-1+ T cells (MFI Control: 187; 1D11: 139.5; shActA: 162; 1D11+shActA: 111; RT: 138.5; RT+1D11: 379; RT+shActA: 182; RT+1D11+shActA: 498.5).

Overall, data indicate that TGFβ and actA regulate Tregs numbers in tumors and show a complex interaction with RT. Combined blockade of TGFβ and actA during RT may be required to improve in situ vaccination by RT.

Citation Format: Claire I. Vanpouille-Box, Silvia C. Formenti, Sandra Demaria. Regulation of radiation-induced in situ tumor vaccination by TGFβ superfamily members. [abstract]. In: Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16-20; New Orleans, LA. Philadelphia (PA): AACR; Cancer Res 2016;76(14 Suppl):Abstract nr 4986.

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Clinical Research (Excluding Clinical Trials)

Abstract 4987: Role of the PD-1/PDL-1 pathway in resistance of patients with metastatic breast cancer to treatment with radiotherapy and TGFβ neutralization

Sandra Demaria, Claire Vanpouille-Box, Rachael E. Hawtin, Christie Fanton, Andy Conroy, Erik Evensen, Neha Dixit, Alan Barber, Dorthe Schaeue, William H. McBride, Mary Helen Barcellos-Hoff, and Silvia C. Formenti
DOI: 10.1158/1538-7445.AM2016-4987 Published 15 July 2016

Article

Info & Metrics

Proceedings: AACR 107th Annual Meeting 2016; April 16-20, 2016; New Orleans, LA

Abstract

Experimental data and clinical observations indicate that local radiation therapy (RT) converts the irradiated tumor into an in situ vaccine and improves responses to immunotherapy. Preclinical breast cancer models show that TGFb neutralization was required to achieve CD8+ T cell priming specific for multiple tumor antigens, and rejection of the irradiated tumor and non-irradiated metastases (Vanpouille-Box et al., Cancer Res 2015). Tumor response and survival were improved by PD-1 blockade. We explored the role of the PD-1/PDL-1 pathway in resistance to combined RT + TGFβ neutralizing antibody (fresolimumab) in metastatic breast cancer patients (pts).

22 pts were treated in a prospective trial at NYU and UCLA (NCT01401062, supported by DOD MTA BC100481). Pts were randomly assigned to 2 doses of fresolimumab (freso) (1mg/kg and 10 mg/kg, q 3 weeks). Image guided RT was delivered to one metastasis on weeks 2 and 7, 7.5 GyX3. 1 pt achieved objective response, with 28% reduction of tumor burden. The response lasted 11 months, anthracycline-induced acute myelogenous leukemia developed. Pts randomized to the higher freso dose had a hazard ratio of 2.17 (95% CI: 0.753-6.272) for risk of death at 1 year follow up. Currently, at a median follow up of 2 years, 20/22 pts have died.

To explore reasons for the limited response, peripheral blood mononuclear cells collected at baseline, 5 and 15 weeks into treatment, were analyzed. Responses to survivin, as a tumor antigen, were assessed using tetramers. Single cell network profiling (SCNP) was used to evaluate expression of 6 immunomodulatory receptors plus immune signaling in T cells collected from 7 healthy donors (HD) and 15 breast cancer pts (6 at 1mg/kg, 9 at 10mg/kg fresolimumab). In vitro activation of p-AKT and p-Erk was quantified following in vitro T cell receptor (TCR) modulation with anti-CD3/anti-CD28.

6/12 HLA-A2.1+ pts had pre-existing survivin-specific CD8+ T cells, and 3 showed an increase over time. 2 pts who were negative generated modest responses after RT and 10 mg/kg freso. Prior to treatment, PDL-1 expression was increased in monocytes (p = 0.01) and CD4+ T cells (p = 0.037) compared to HD. Elevated PD-1 (p = 0.054) and OX-40 (p = 0.014) expression were identified on

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CD4+ T cells of pts. Upon TCR modulation, CD4+ and CD8+ T cells from pts showed reduced signaling through p-AKT and, to a lesser extent, p-Erk, compared to HD. Signaling was lower in PD-1+ vs PD-1- CD8+ and CD4+ T cells. Addition of anti-PD-1 pembrolizumab partially restored TCR-mediated signaling through p-AKT and p-Erk in PD-1+ but not PD-1- T cells.

Overall, data indicate impaired T cell signaling in PD-1+ T cells of metastatic breast cancer pts, which may explain the inability to respond to RT + TGFβ blockade. In vitro addition of anti-PD-1 improved T cell signaling through p-AKT and p-Erk. Together with the preclinical data, this supports adding anti-PD-1 to the combination of RT +TGFβ blockade.

Citation Format: Sandra Demaria, Claire Vanpouille-Box, Rachael E. Hawtin, Christie Fanton, Andy Conroy, Erik Evensen, Neha Dixit, Alan Barber, Dorthe Schaeue, William H. McBride, Mary Helen Barcellos-Hoff, Silvia C. Formenti. Role of the PD-1/PDL-1 pathway in resistance of patients with metastatic breast cancer to treatment with radiotherapy and TGFβ neutralization. [abstract]. In: Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16-20; New Orleans, LA. Philadelphia (PA): AACR; Cancer Res 2016;76(14 Suppl):Abstract nr 4987.

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Suppression of Major Histocompatibility Complex (MHC) Class I and II Mediates Resistance to Anti-PD-1 in Lung Adenocarcinoma Tumors That Can Be Overcome by Radiation Therapy

X. Wang,¹ J.E. Schoenhals,¹ D.R. Valdecana,¹ A. Li,¹ H. Ye,² F. Zhang,³ M. Tang,⁴ C. Tang,⁵ C.G. Liu,⁶ X. Liu,⁶ R.U. Komaki,⁵ D.R. Gomez,⁵ J.Y. Chang,⁵ M.A. Cortez,¹ and J.W. Welsh⁵; ¹Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, ²Department of Experimental Radiation Oncology, MD Anderson Cancer Center, Houston, TX, ³Department of Pathology The University of Texas MD Anderson Cancer Center, Houston, TX, ⁴Department of Genomic Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, ⁵Department of Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, ⁶Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX

Purpose/Objective(s): PD-1/PD-L1 blockage therapy, given as single agent have produced objective response rates ranging from 15% to 25% in patients with chemotherapy refractory non-small-cell lung carcinoma (NSCLC). Nevertheless, large proportions of patients do not respond to anti-PD-1/PD-L1 immunotherapies. Therefore, we sought to investigate the mechanisms of nonresponses and potential strategies to overcome resistance. Here we generated an anti-PD-1-resistant preclinical tumor model with which to identify mechanisms of resistance, which should provide novel insights to expand the benefit of immunotherapies.

Materials/Methods: This model was generated by inoculating the murine lung cancer cell line 344SQ (containing p53R172H-g/+K -rasLA1/+ mutations) into syngeneic 129Sv/ev mice, which were then given 4-5 doses of anti-mouse PD-1 antibody (10 mg/kg). The anti-PD-1 resistant tumor model was successfully generated after sequential in vivo passage of a nonresponsive tumor with ongoing anti-PD-1 treatment. For the combined radiation plus anti-PD-1 therapy studies, the first dose of anti-PD-1 (10 mg/kg) was given on the same day of first fraction of radiation (12G in 3 fractions) and continued for additional 3-4 doses. For experiments involving blockade of type I IFN signaling, anti-mouse IFNAR-1 antibody was injected daily for 14 days, starting on the day of first dose of anti-PD-1. For studying the growth rate of the tumor mass, the length (L) and width (W) of tumors were measured with calipers. Tumor volume (V) was calculated as: $V = W^2 \times L/2$. The unpaired Student t test was used for analysis of most data except that the tumor growth curve was analyzed by multiple t tests for each time points. All reported P values are two-sided and were declared as significant at the level of 5%.

Results: PD-L1 expression was not different in the resistant vs. parental tumor cells. Microarray and flow cytometry studies demonstrated that genes in the antigen presentation pathway, including major histocompatibility complex (MHC) class I and II, were significantly down-regulated in the anti-PD-1-resistant tumors compared with parental tumors. Resistant tumors also showed fewer CD8+ and CD4+ tumor-infiltrating lymphocytes and reduced production of interferon (IFN) γ . Local tumor radiotherapy induced IFN γ production, which in turn induced MHC class I expression on both parental and resistant tumor cells and restored the resistant tumor to anti-PD1 response. Blockade of type I IFN signaling abolished the effect of radiation on sensitization of anti-PD-1 response.

Conclusion: We discovered a novel mechanism of PD-1 resistance and demonstrated radiation can be used to overcome such resistance. Our findings suggest that the poor efficacy of anti-PD1 as an immune checkpoint therapy in most patients might be accentuated greatly by adjuvant radiotherapy, thereby broadening its useful application against melanoma and lung cancers where robust but relatively infrequent responses have been documented.

Author Disclosure: X. Wang: None. J.E. Schoenhals: None. D.R. Valdecana: None. A. Li: None. H. Ye: None. F. Zhang: None. M. Tang:

None. C. Tang: None. C. Liu: None. X. Liu: None. R.U. Komaki: None. D.R. Gomez: travel costs and speaker; ProCure. J.Y. Chang: Shareholder; Varian Medical System. Director; The University of Texas MD Anderson Cancer Center. M.A. Cortez: None. J.W. Welsh: Laboratory research support; Varian, GSK, Mirnatherapeutics, Incyt, Merck. Clinical research support; BMS, Merck. Scientific Advisor; Reflexion Medical, Checkmate Pharmaceuticals. Cofounder and officer; Molecular Match. Cofounder; Healios, OncoResponse.

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TGF β Superfamily Members Regulate Radiation-Induced In Situ Tumor Vaccination

C. Vanpouille-Box, S. Formenti, and S. Demaria; Weill Cornell Medical College, New York, NY

Purpose/Objective(s): Transforming Growth Factor-beta (TGF β) and activin A (actA) are members of the TGF β superfamily with overlapping as well as distinct functions in many processes including regulation of inflammation and immunity. Similar to TGF β , actA has been shown to promote the conversion of CD4+ CD25- T cells into induced regulatory T cells (Tregs) and to potentiate iTregs generation by TGF β . Many cancer cells produce actA, and we have recently found that actA released in vitro by breast cancer cells are enhanced by the radiation-induced DNA-damage response. Interestingly, prolonged exposure to TGF β inhibitors also resulted in enhanced actA release, consistent with a compensatory mechanism described by Carvalho et al., J Cell Sci, 2007. Here we tested the hypothesis that both actA and TGF β play a critical role in regulating anti-tumor immunity induced by radiotherapy (RT).

Materials/Methods: The 4T1 derivatives with conditional actA knock-down (4T1^{shActA}) or non-silencing control (4T1^{shNS}) were engineered using inducible doxycycline (DOX) plasmids and injected s.c. in BALB/c mice (day 0). ActA gene knockdown (KD) was induced by DOX at day 8. TGF β neutralizing 1D11 or isotype control antibodies were given i.p. every other day starting on day 12. RT was delivered to the primary tumor in 6 Gy fractions on five consecutive days starting at day 13. Mice were followed for tumor growth and survival or euthanized at day 22 and day 28 for analysis.

Results: Neither 1D11 nor ActA gene KD by themselves inhibited tumor growth. However, each intervention significantly improved tumor control achieved by RT. ActA KD in mice treated with RT + 1D11 prevented tumor recurrence and improved survival (RT + 1D11 vs RT + 1D11 + shActA, $P = 0.026$; RT + shActA vs RT + 1D11 + shActA, $P = 0.0008$). Interestingly, blockade of TGF β or actA resulted in increased intratumoral Tregs (Control: 11.6%; 1D11: 26.2%, shActA: 21%) and markedly enhanced the increase in Tregs seen with RT alone (RT: 15.7%; RT + 1D11: 27.5%; RT + shActA: 30.3%). In contrast, when both TGF β and actA were inhibited Tregs significantly decreased in both non-irradiated (1D11 + shActA: 13.6%) and irradiated tumors (RT + 1D11 + shActA: 7.9% of Tregs). IFN γ production by CD8+ T cells in response to the tumor-specific AH1 peptide was significantly higher in RT + 1D11 + shActA treated mice compared to RT + 1D11 ($P = 0.045$) and RT + shActA ($P = 0.0147$). Additionally, in RT + 1D11 + shActA mice expression of the activation marker CD69 was markedly increased in intratumoral CD8+ PD-1+ T cells (MFI Control: 187; 1D11: 139.5; shActA: 162; 1D11 + shActA: 111; RT: 138.5; RT + 1D11: 379; RT + shActA: 182; RT + 1D11 + shActA: 498.5).

Conclusion: Overall, data indicate that both TGF β and actA attenuate the responsiveness of the immune system after RT. Concomitant inhibition of actA and TGF β during RT may promote self-immunization and achieve systemic control of metastatic disease.

Author Disclosure: C. Vanpouille-Box: None. S. Formenti: Research Grant; Regeneron Pharmaceuticals, Janssen Biotech, Eli Lilly. Speaker's Bureau; Varian. Advisory Board; BMS, Smith Kline, AstraZeneca, and Eisai. S. Demaria: Advisory Board; Regeneron Pharmaceuticals, Snafu US Inc.

P260 ATM is essential for the radiation-induced upregulation of the immunosuppressive cytokine activin-A by breast cancer cells

Claire I Vanpouille-Box, Silvia C Formenti, Sandra Demaria

Weill Cornell Medicine, Department of Radiation Oncology, New York, NY, USA

Correspondence: Claire I Vanpouille-Box (clv2002@med.cornell.edu)

Background

Activin A (actA) is a member of the transforming growth factor beta (TGF β) superfamily. Recent evidence suggests that actA may facilitate tumorigenesis in part by suppressing immunity in the tumor microenvironment [1]. Treatment-induced DNA double-strand breaks (DSBs) induce actA mRNA and protein [2]. The ataxia telangiectasia-mutated (ATM) kinase is activated at DNA DSBs caused by genotoxic agents such as radiotherapy (RT) and is critical for DNA repair. Here we tested the hypothesis that induction of actA by RT limits RT-induced activation of anti-tumor immunity.

Methods

To test this hypothesis, 4 T1 mammary carcinoma cells were engineered to express a doxycycline (dox) inducible shRNA silencing inhibin A (*Inhba*, gene encoding for actA) (4T1^{shInhba}). 4T1^{shInhba} or its non-silencing control (4T1^{shNS}) were exposed to ionizing radiations to determine *Inhba* gene expression by RT-qPCR as well as secretion of actA by ELISA. To determine if ATM controls the expression of actA, derivatives with inducible knockdown of ATM were also generated (4T1^{shATM}). 4T1^{shInhba}, 4T1^{shATM} and 4T1^{shNS} were injected s.c. in syngeneic BALB/c mice on day 0. Knockdown of *ATM* and *Inhba* genes was induced by dox at day 8. Tumors were irradiated with 6 Gy repeated on days 13, 14, 15, 16 and 17. Mice were monitored and euthanized at day 22 and day 28 for evaluation of immune cells infiltration into the tumor.

Results

RT upregulated actA expression and secretion by 4 T1 cells. Secreted actA promoted CD4⁺ T cells conversion into regulatory T (Tregs) cells. *In vitro*, knockdown of ATM abolished both *Inhba* gene expression and actA secretion by tumor cells after RT. *In vivo*, this resulted in reduced Tregs infiltration in irradiated tumors, and increased activation of intra-tumoral CD8⁺ T cells. 4T1^{shInhba} and 4T1^{shATM} tumors showed an increased response to RT compared to 4T1^{shNS} tumors.

Conclusions

These data suggest that ATM plays a critical role in RT-induced actA secretion, which promotes an immunosuppressive environment in the irradiated tumor. Inhibition of ATM may increase tumor radiosensitivity and at the same time enhance *in situ* vaccination by radiation by hindering Treg generation.

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